Optimization of bacteriocin production by batch fermentation of *Lactobacillus plantarum* LPCO10

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ABSTRACT

Optimization of bacteriocin production by *Lactobacillus plantarum* LPCO10 was explored using an integral statistical approach. In a prospective series of experiments, glucose and NaCl concentrations in the culture medium, inoculum size, aeration of the culture, and temperature of growth were statistically combined following an experimental $2^{3}_{(5-2)}$ fractional factorial two-level design and tested for their influence on maximal bacteriocin production by *L. plantarum* LPCO10. After fixing the values for the lesser influential variables, NaCl concentration, inoculum size and temperature were further selected to study their optimal relationship for maximal bacteriocin production. This was achieved by a new experimental $3^{2}_{(3-1)}$ fractional factorial three-level design which was subsequently used to build the respective response surfaces and analyzed for both linear and quadratic effects. Results obtained indicated that the best conditions for bacteriocin production were shown with temperature ranging from 22 to 27 ºC, salt concentration from 2.3 to 2.5%, and *L. plantarum* LPCO10 inoculum size ranging from $10^{7.3}$ to $10^{7.4}$ CFU/ml, fixing the initial glucose concentration at 2%, with no aeration of the culture. Under these optimal conditions, about 3.2×10^{4} times more bacteriocin per litre of culture medium was obtained than that used to initially purify plantaricin S from *L. plantarum* LPCO10 to homogeneity. These results indicated the importance of this study to obtain bacteriocins from *L. plantarum* LPCO10 to be applied in canned foods.

INTRODUCTION

Lactic acid bacteria (LAB) are widely used as starter cultures in dairy, meat, and vegetable fermentations (1, 7, 37). One of the major reasons for this is the wide range of antimicrobial substances which they are able to produce, efficiently contributing to the preservation of the fermented products (13, 15, 31). Among those, bacteriocins are one of the most promising natural food preservatives produced by LAB (10, 12, 26, 33, 34). This preservation potential could be achieved either by using a bacteriocin producing starter culture or applying the bacteriocin itself as a food additive. The latter will necessarily require optimization of their production, for this is dependant on multiple factors which usually are strain specific (9, 40).

In the past, several studies have pursued this goal for a number of different bacteriocins. They have generally focused on the effects of pH, temperature, composition of the culture media, and general microbial growth conditions, *in vitro* as well as in natural fermentations, over maximal bacteriocin production (2, 4, 6, 8, 11, 14, 24, 25, 28-30, 39-41; among others). However, although some of these studies claim validation by a statistical test, usually variance analysis, combination of variables as well as their values and limits are arbitrarily chosen, based mainly on subjective personal experience. Thus, no previous prospective experimental design is usually carried out in order to optimize the information that can be gained from the subsequent experiments.

In this work, we explore the valuable power of statistical experimental design to optimize the production of bacteriocins by *Lactobacillus plantarum* LPCO10 (21, 22, 24,

36) in order to be applied as natural food additives in canned vegetables and other food systems.

MATERIALS AND METHODS

Bacterial strains. *Lactobacillus plantarum* LPCO10 (plantaricins S [PLS] and T [PLT] producer) and *L. plantarum* 128/2 (PLS and PLT sensitive) belong to our own culture collection and have been previously described (21). *L. fermentum* ATCC 14933 (PLS- and PLT-sensitive) was obtained from the CNRZ Culture Collection, Jouy-en-Josas, France.

Culture media. For maintainance purposes and bacteriocin sensitivity assays, the strains were grown at 30 ºC in MRS medium (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England). For the optimization experiments, MRS broth prepared in our own laboratory was used which contained (in grams per litre of deionized distilled water): peptone (E. Merck, Darmstadt, Germany), 10.0; Lab-Lemco powder (Oxoid), 8.0; yeast extract (Oxoid), 4.0; K_2HPO_4 (Fluka Chemie AG, Buchs, Switzerland), 2.0; $NaCH_2COOH \cdot 3H_2O$ (Fluka), 5.0; di-ammonium hydrogen citrate (Fluka), 1.84; $MgSO_4 \cdot 7H_2O$ (Merck), 0.2; $MnSO_4 \cdot H_2O$ (Merck), 0.04; and Tween 80 (Fluka), 1 ml. When required, 3 to 5% (w/vol) NaCl (Merck) was added, and the pH was adjusted to 6.2 with 12N HCl (Fluka). The volume was adjusted to 950 ml with deionized distilled water and then esterilized by autoclaving at 121 ºC. Fifty percent (w/v) filter-sterilized glucose (Fluka) was added to bring the final glucose concentration to 1 or 2%, and the final volume of the medium was brought to 990 ml by the addition of sterile distilled water. The medium was brought to final volume before each experiment by addition of 10 ml of the *L. plantarum* LPCO10 inoculum in sterile saline.

Inoculum preparation. One single colony of *L. plantarum* LPCO10 growing in MRS agar (Oxoid) was inoculated into 20 ml of MRS broth, prepared as above, containing 2% glucose, and brought up to the final volume with sterile water. It was incubated at 30 ºC overnight and then used to inoculate 2 litres of fresh MRS medium. After incubation at 30 ºC, cells were collected by centrifugation (5,000 × *g*, 10 min) when the absorbance at 600 nm (A_{600nm}) reached 0.7 (ca. 10^8 CFU/ml), washed with sterile saline and resuspended in 200 ml of 15% (vol/vol) glycerol. Aliquots were made and stored at –80 ºC until use.

Fermentation experiments. A 2-litre Biostat MD fermenter (B. Braun-Biotech, Melsungen, Germany) containing one litre of the above described MRS medium was used to study the kinetics of bacteriocin production by *L. plantarum* LPCO10. For every experiment, one of the above generated stock aliquots was thawed and the neccessary amount of cells were collected by centrifugation (5,000 × *g*, 10 min), washed and finally resuspended in 10 ml of sterile saline, which was then used to inoculate the fermenter. When appropriate, aeration was set by bubbling filter-sterilized air at a rate of 1 l/min. Cultures were stirred at 40 rpm, throughout the fermentations.

Analysis of the samples. At convenient time intervals, samples were aseptically withdrawn from the fermentation vessel to determine glucose concentration, viable cell number, bacteriocin activity, and lactic acid production. Furthermore, pH value was monitored on line by the fermenter probe. Glucose concentration was determined by the D-glucose/D-fructose Enzymatic BioAnalysis kit from Boehringer Mannheim GmbH (Mannheim, Germany), according to the manufacturers instructions. Viable cell counts were determined by plating serial dilutions of the samples onto MRS agar (Oxoid) by a Spiral Systèm (Interscience, Saint-Nom-La Bretèche, France) and incubating at 30 ºC for 48 h. Bacteriocin activity was quantified with a microtiter plate assay system as described

before (19, 22), using *L. plantarum* 128/2 and *L. fermentum* ATCC 14933 as the indicator strains, averaging two- and threefold dilution series for every sample (17, 22), and expressed as arbitrary activity units per ml (AU/ml). Lactic acid concentration (expressed as mmol/litre) was determined acidimetrically by titration with 0.1 N NaOH to an endpoint pH of 6.8.

Experimental design. Initially, responses, i.e. inhibitory activity due to bacteriocin production by *L. plantarum* LPCO10, were detected by using *L. plantarum* 128/2 and *L. fermentum* ATCC 14933 as indicator cultures. Bacteriocin production was measured as the $\log_{10}$ of AU/ml, and it was assumed to be under the influence of the glucose concentration, the inoculum size, aeration, temperature, and NaCl concentration. Their effects were tested in the diagnostic experiment by the $2^{3}_{(5-2)}$ fractional factorial two-level design shown in Table 1A (5, 23). In this design, column fourth (temperature) was obtained by combination of columns one and two (glucose concentration, and aeration of the culture, respectively), and column five (NaCl concentration) was obtained by combination of columns two and three (inoculum size, and aeration, respectively). Physical and coded levels are also shown in Table 1B. In general, coded values for continuous variables were obtained as follows:

coded value= [physical value- ½ (highest value + lowest value)]/ ½ (highest value – lowest value).

In case of qualitative variables, coded values were assigned at random. All experiments were randomly run. As responses, the logarithm of maximum bacteriocin activity detected in the supernatants for every fermentation run was used. It was a measure of the total inhibitory activity that can be collected at that sampling point, and

represents the maximal bacteriocin production for that fermentation run. Moreover, two responses were obtained for every single experiment, one for each indicator strain used to titrate the bacteriocin activity. The logarithmic transformation was used to reduce variance due to the wide range of activity values obtained.

After the analysis of this previous experiment, a new three-level $3^{2}_{(3-1)}$ fractional factorial design (35) with replication at the central point was performed (Table 2). Values of the variables under study were selected to include the region of highest bacteriocin activity, as concluded from the diagnostic experiments (see Results). The responses considered were the same as in the previous diagnostic design. Results were analyzed for lineal and quadratic effects and used to build the corresponding response surfaces.

Statistical analysis. Results were analysed by the Experimental Design Module of the Statistica software package (35).

RESULTS

Diagnostic experimental design of bacteriocin production by *L. plantarum* LPCO10. When the effects of the individual factors (glucose concentration, inoculum size, aeration of the culture, temperature of incubation, and NaCl concentration) were assessed, none of them showed any statistically significant effect on bacteriocin production by *L. plantarum* LPCO10 when *L. plantarum* 128/2 was used to analyze responses (bacteriocin production). Thus, due to the limitations of the design, no information on the response in the region of the design could be obtained. However, when *L. fermentum* ATCC 14933 was used, temperature as well as NaCl concentration were found to have significant ($p < 0.05$) effects on the responses (Figure 1). The sign of these estimated effects was negative in both cases, thus indicating that the response decreased when the temperature or the NaCl concentration increased, and conversely, into the limits of the coded values used in this study (Table 1B). On the other hand, glucose concentration, inoculum size, and aeration did not show any significant effect on the responses ($p < 0.05$) (Figure 1). However, the sign of the effects of the two first was positive, indicating that to an increase in glucose concentration or in the inoculum size would follow only a slight favourable effect on the response within the limits studied.

A predictive estimation of the expected bacteriocin production by *L. plantarum* LPCO10 at the limit coded values of temperature and NaCl concentration showed that the highest titers of bacteriocins would be obtained when the values of these parameters were around 25 ºC and 3% NaCl (data not shown), whatever the values of the other parameters studied, within the limits described in Table 1B. Actually, run no. 4, which was

the one obtaining the highest responses (Table 1A), was repeated to confirm the results. Responses were again, as expected, the highest (data not shown).

The purpose of this diagnostic two-level design was to obtain experimental data which served as a first approach to final optimization of bacteriocin production, establishing which factors had significant effects on responses (temperature and NaCl concentration, in this case) and the sign of these effects. With respect to the other variables, their levels did not statistically affect the responses. However, there were slightly lower responses in the case of aeration and, on the other hand, slightly higher responses were obtained when 2% glucose or 10^7 CFU/ml were used. In consequence, in further experiments, aeration was not applied, the glucose level was fixed at 2%, and the inoculum size tested again to investigate a possible correlation between bacterial growth and bacteriocin production.

Study of quadratic effects and response surfaces for maximal bacteriocin production by *L. plantarum* LPCO10. To estimate not only linear but also quadratic effects and build response surfaces for maximal bacteriocin production in the region defined by the previous diagnostic experiments, a new experimental design at three levels was planned and the corresponding experiments were carried out. For this, aeration was omitted and initial glucose concentration in the media was fixed at 2%. Temperature, NaCl concentration and inoculum size were selected as the variables (Table 2). Coded values for these variables were different from those used in the exploratory experiment and the physical levels were selected taking into account the results from the diagnostic design. Thus, the limits for some variables were reduced or expanded, according to the previous results: temperature and NaCl concentration were lowered, while the limit for inoculum size was increased (Table 2B). The central values (coded value = 0) were those

found to promote the highest bacteriocin production in the previous diagnostic round of experiments for the corresponding variable.

Responses obtained now were again different depending on the bacteriocin sensitive strain used (Table 2A), as well as on the effects of every variable within the new limits. In general, responses obtained with *L. plantarum* 128/2 were higher than those obtained in the exploratory experiments which allowed the detection of effects and the application of the statistical analyses. Thus, when *L. plantarum* 128/2 was used as an indicator strain, NaCl concentration (lineal) and inoculum size (lineal and quadratic) were found to have significant ($\rho < 0.05$) effect on the response (bacteriocin production). The corresponding response surface is shown in Figure 2, where a point for maximum bacteriocin production can be found. The conditions corresponding to this point can be easily deduced from the contour lines at the bottom of Figure 2 (plane NaCl concentration-$\log_{10}$ inoculum size), and are defined by NaCl concentration of 2.5% and $10^{7.3}$ CFU/ml of *L. plantarum* LPCO10. On the other hand, temperature did not show any significant effect in the interval considered for this variable in this second design. So, the lowest limit might be used since their energetic requeriments are lower.

When *L. fermentum* ATCC 14933 was used as the indicator strain, the responses were again higher than those obtained with the 128/2 strain, although response trends maintain similar proportionalities in all runs (Table 2A). No new significant effects of the variables were found in this second round of experiments, indicating that the region of optimal conditions, as detected by strain ATCC 14933, had already been included in the diagnostic design. However, the same variables still showed the highest influence, as it is shown by the response surfaces that can be obtained for every parameter combination regarding strain ATCC 14933 responses. Thus, the relationship between temperature and

NaCl concentration is shown in Figure 3. As it was found with *L. plantarum* 128/2, temperature has a negligible effect in the interval selected now (from 22 to 27 ºC), as it is shown by the virtually horizontal line corresponding to the temperature axis. On the other hand, NaCl concentration showed a stimulating influence on the response up to 2.5% (as with strain 128/2), but concentrations above this level tend to decrease bacteriocin production.

The relationship between NaCl concentration and inoculum size is shown in Figure 4. From the contour lines represented in this Figure, it is easily deduced that maximal bacteriocin production could be obtained with a combination of 2.5% NaCl and $10^{7.4}$ CFU/ml of *L. plantarum* LPCO10, again virtually the same result as with *L. plantarum* 128/2.

Finally, the relationship between temperature and inoculum size (Figure 5) points out again the negligible influence of temperature within the proposed limits, as well as the fact that when the inoculum size of *L. plantarum* LPCO10 is over $10^{7.4}$ CFU/ml there is no further increase in bacteriocin production, but even a slight decrease.

Relationship between bacteriocin production and growth of *L. plantarum* LPCO10. It could be argued that conditions for maximal bacteriocin production should correspond to those conditions allowing also maximal cell density of the producer strain, as bacteriocin is usually a primary metabolite (11, 14, 25). Thus, the three variables found to have a significant effect on bacteriocin production (NaCl concentration, temperature and inoculum size), and whose relationship has been optimized, also showed a significant linear effect on the maximal *L. plantarum* LPCO10 cell density. This is shown by the respective response surfaces obtained by having now the $\log_{10}$ of CFU/ml at the points of

maximal bacteriocin production in every run as the response. These response surfaces are very simple, virtually planes, indicating that there is no correlation between both responses (log_{10} of maximal bacteriocin activity produced and log_{10} of CFU/ml at the same points) in the interval of values assayed for the variables under study. As an example, Figure 6 shows the relationship between NaCl concentration and temperature when the response is now the CFU/ml of the LPCO10 strain at the points of maximal bacteriocin production. This figure just shows that the cell density decreases linearly as the temperature increases, and decreases as the NaCl concentration increases (although with a lower slope).

DISCUSSION

Optimization of bacteriocin production by *L. plantarum* LPCO10 in a lab fermenter, as preliminary step to production of bacteriocin for use as a preservative in canned foods (mainly vegetables), was acomplished. As high amounts of bacteriocin are necessary to test their preservative efficiency in natural environments (2, 10, 13, 33, 37), establishment of factors and their levels influencing maximal production would lead to a more effective recovery of these antimicrobial compounds from a defined laboratory culture medium.

The study was accomplished in two consecutive steps: first, a diagnostic two-level design was carried out to determine the variables that could affect bacteriocin production by *L. plantarum* LPCO10; later, a $3^{2}_{(3-1)}$ fractional factorial three-level design with replication at the central point was carried out to estimate linear and quadratic effects, as well as the corresponding response surface. In general, responses obtained using *L. plantarum* 128/2 as the indicator strain were always lower than those obtained with *L. fermentum* ATCC 14933: this difference averaged two log units, and the behaviour was similar in all runs. This was an expected result, for *L. fermentum* ATCC 14933 has always shown to be more sensitive than *L. plantarum* 128/2 to PLS and PLT (21). Sensitivity to the bacteriocins is apparently an intrinsic characteristic of each strain and does not depend on the conditions of the culture media from which bacteriocin was obtained. Thus, an interesting conclusion of the first part of this study was the need for selecting an appropriate bacteriocin indicator strain. If related studies on optimization of bacteriocin production are to be developed using statistical approaches, it seems advisable, if not absolutely necessary, to select those strains which are most sensitive to the bacteriocin

under study. In other terms, strains must be selected which can offer the highest responses. In our case, for the parameters and the limit values used, results obtained from *L. fermentum* ATCC 14933 showed better the effects than *L. plantarum* 128/2, although both of them are quite sensitive to the bacteriocins studied. An adequate quantitative titration method for inhibitory activity is also necessary. We suggest using the microtiter plate method described previously (19), averaging two- and three-fold dilutions series for every sample (17, 22).

The second part of this study indicates that the best conditions for bacteriocin production by *L. plantarum* LPCO10 (in the fermenter and the culture medium described here) are at about 2.5% NaCl concentration, temperature ranging from 22 to 27 ºC, and inoculum size of about $10^{7.4}$ CFU/ml, with no aeration and initial glucose concentration between 1 and 2%. Interestingly, these results clearly correlate with the empiric way by which the olives are processed. In this traditional fermentation, the fruits are covered with a brine around 5-6% NaCl (final concentration). The containers of about 10 tons of fruits and 5,000 litres of brine are placed outdoor, therefore many times the fermentation is carried out at temperatures ranging from 20 to 25 ºC (16, 18). Also, aeration is not a usual practice in the green olive fermentation, and concentration of sugars in the fruits is estimated to be about 2 to 6% the pulp weight, therefore there is no carbon nutrients limitation for lactobacilli. On the other hand, the strain *L. plantarum* LPCO10 used in this work, originally isolated from a green olive fermentation (21), has been successfully used as a starter culture for olive fermentations previously (24, 34). In these cases, it has been clearly demonstrated that the strain dominated the epiphytic microflora and that this domination was due to the ability of *L. plantarum* LPCO10 to produce bacteriocin (34). Thus, the statistical study applied here, even if a synthetic culture medium has been used, generates results which resemble those obtained at the natural environment of *L.*

plantarum in green olive fermentations. This paralelism could indicate that the best conditions established here for maximal bacteriocin production by *L. plantarum* LPCO10 in a lab medium perhaps are quite similar to the best ones for bacteriocin production in the natural olive fermentations. Moreover, results recently obtained by us (M.V. Leal-Sánchez, J.L. Ruiz-Barba, A.H. Sánchez, L. Rejano, R. Jiménez-Díaz, and A. Garrido, submitted for publication) indicate that the traditinal fermentation of Spanish-style green table olives could be damatically improved by using *L. plantarum* LPCO10 as a starter culture at $\geq 10^7$ CFU/ml (final concentration in brines), and a starting brine of 4% NaCl. These results obtained at an experimental, semi-industrial level again correlate with those conditions obtained in this study for maximal bacteriocin production by *L. plantarum* LPCO10 at a laboratory level.

On the other hand, results obtained indicate that there is not a clearcut correlation between maximal bacteriocin production and the cell number of *L. plantarum* LPCO10 at the same points. However, it has been shown here that at the interval points of maximal bacteriocin production the cell density decreases as the temperature increases, and decreases as the NaCl concentration increases (Fig. 6). From this, it can be concluded that maximal production of bacteriocin by *L. plantarum* LPCO10 is not only related to the cell number, but to additional factors. For instance, salt concentration have been found to play an important role in the release of bacteriocins from different bacteriocin producing strains of lactic acid bacteria during growth, most probably by influencing the adsorption of the bacteriocins to the cell envelope of the bacteriocin producer itself (3, 20, 21, 25, 27, 32, 38).

Finally, when *L. plantarum* 128/2 was used as the indicator strain, maximal bacteriocin production by *L. plantarum* LPCO10 is shown by conditions described in run

no. 10 (3,200 AU/ml; Table 2). This amount of bacteriocin is about 3.2×10^4 times the amount from which purification to homogeneity of PLS was achieved, using the same 128/2 strain as the sensitive strain to monitor the purification steps (22). Thus, results obtained here clearly indicated the importance of this study in the production of bacteriocins from *L. plantarum* LPCO10 for application in canned foods.

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LEGENDS OF THE FIGURES

Figure 1. Pareto chart of standardized effects of the different variables tested in the prospective experiment on bacteriocin production by *L. plantarum* LPCO10. *Lactobacillus fermentum* ATCC 14933 was used as an indicator strain. Symbols: T= temperature; S= NaCl concentration; G= initial glucose concentration; IS= initial inoculum size; A= aeration). The point at which the effect estimates were significant at p = 0.05 is indicated by the vertical solid line.

Figure 2. Response surface of bacteriocin production by *L. plantarum* LPCO10, estimated as the $\log_{10}$ of maximal arbitrary activity units per ml (AU/ml), and contour lines showing the optimum region, as a function of NaCl concentration and the *L. plantarum* LPCO10 inoculum size. *Lactobacillus plantarum* 128/2 was used as the indicator strain.

Figure 3. Response surface of bacteriocin production by *L. plantarum* LPCO10, estimated as the $\log_{10}$ of maximal arbitrary activity units per ml (AU/ml), as a function of temperature and NaCl concentration. *Lactobacillus fermentum* ATCC 14933 was used as an indicator strain.

Figure 4. Contour plot showing the optimum region of bacteriocin production by *L. plantarum* LPCO10, estimated as the $\log_{10}$ of maximal arbitrary activity units per ml (AU/ml), as a function of the inoculum size and NaCl concentration. *Lactobacillus fermentum* ATCC 14933 was used as the indicator strain.

Figure 5. Response surface of bacteriocin production by *L. plantarum* LPCO10, estimated as the $\log_{10}$ of maximal arbitrary activity units per ml (AU/ml), and contour lines showing the optimum region, as a function of temperature and initial inoculum size. *Lactobacillus fermentum* ATCC 14933 was used as the indicator strain.

Figure 6. Response surface of *L. plantarum* LPCO10 cell density ($\log_{10}$), as a function of NaCl concentration and temperature.

Table 1. Fractional factorial($2^{3}_{(5-2)}$) diagnostic experimental design (A), and coded values (B) used to test the effects of several fermentation parameters over the production of bacteriocin by *L. plantarum* LPCO10. Responses obtained with every run are also indicated in A, depending on the indicator strain used [*L. plantarum* 128/2 **(a)** or *L. fermentum* ATCC14933 **(b)**]. Responses represent the logarithm of maximal bacteriocin activity in that run [$\log_{10}$ (max AU/ml)].

A

Run #	Glucose	Initial inoculum size	Aeration	Temperature	NaCl	Responses	
						(a)	(b)
1	-1	-1	-1	+1	+1	0.0*	2.44
2	+1	-1	-1	-1	+1	2.90	5.05
3	-1	+1	-1	+1	-1	2.44	4.10
4	+1	+1	-1	-1	-1	3.35	7.03
5	-1	-1	+1	-1	-1	3.35	5.28
6	+1	-1	+1	+1	-1	2.14	3.95
7	-1	+1	+1	-1	+1	2.60	4.82
8	+1	+1	+1	+1	+1	1.30	2.74

B

Factors (units)	Actual factor levels at coded values of:	
	-1	+1
Glucose (%, w/vol)	1	2
Inoculum size ($\log_{10}$ CFU/ml)	5	7
Aeration (1 l/min)	yes	no
Temperature (ºC)	25	35
NaCl (%, w/vol)	3	5

*No bacteriocin activity was detected with this strain in this run.

Table 2. Experimental design (A), and coded values (B) used to obtain a response surface of the maximal bacteriocin production by *L. plantarum* LPCO10 as a function of NaCl concentration, temperature and inoculum size. Responses obtained with every run are also indicated in A, depending on the indicator strain used [*L. plantarum* 128/2 **(a)** or *L. fermentum* ATCC 14933 **(b)**]. Responses represent the logarithm of maximal bacteriocin activity in that run [$\log_{10}$ (max AU/ml)].

A

Run #	Temperature	NaCl	Initial inoculum size	Responses	
				(a)	(b)
1	-1	-1	-1	2.90	3.95
2	-1	0	+1	3.35	4.55
3	-1	+1	0	3.05	4.15
4	0	-1	+1	3.05	4.45
5	0	0	0	3.35	4.55
6	0	+1	-1	1.85	3.65
7	+1	-1	0	3.35	4.55
8	+1	0	-1	2.75	3.95
9	+1	+1	+1	2.90	4.41
10	0	0	0	3.51	5.05

B

Factors	Actual factor levels at coded values of :		
	-1	0	+1
Temperature (ºC)	22	25	27
NaCl (%, w/vol)	1	3	5
Initial inoculum size ($\log_{10}$ CFU/ml)	6	7	8

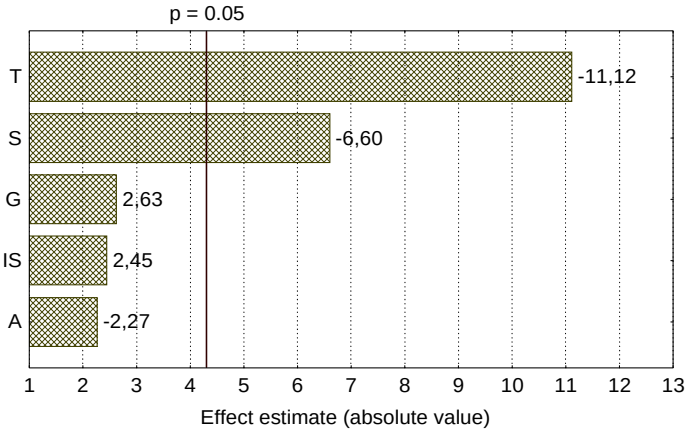

Fig. 1. Leal-Sánchez *et al*.

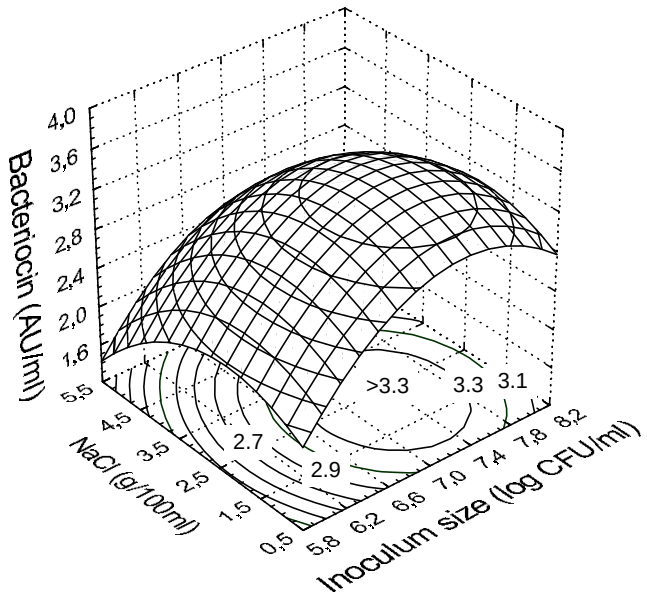

Fig. 2. Leal-Sánchez *et al*.

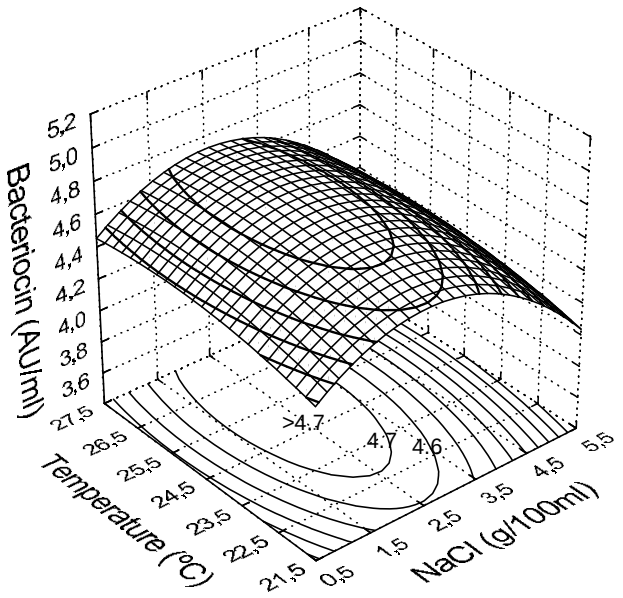

Fig. 3. Leal-Sánchez *et al*.

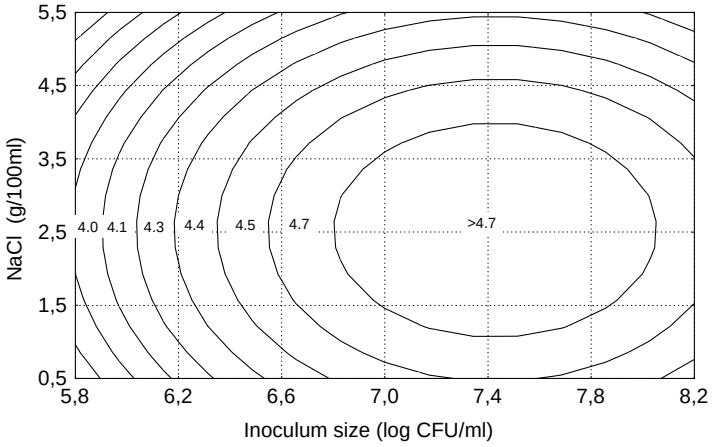

Fig. 4. Leal-Sánchez *et al*.

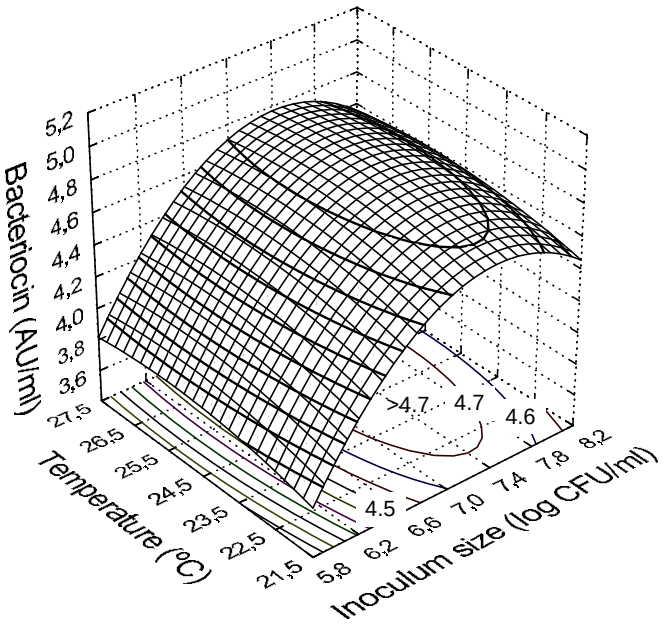

Fig. 5. Leal-Sánchez *et al*.

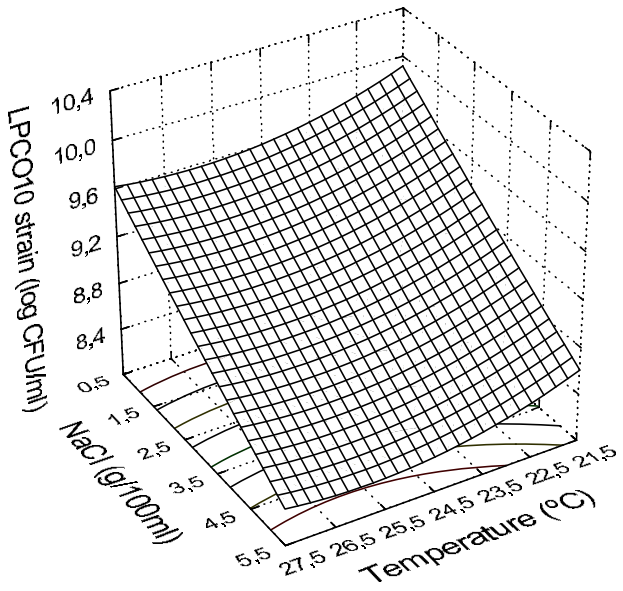

Fig. 6. Leal-Sánchez *et al*.